Plant natural products as insect steroid receptor agonists and antagonists†

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Abstract: Findings to date on plant secondary compounds which possess ecdysteroid-like or anti-ecdysteroid activities in an efficient and effective bioassay based on an ecdysteroid-responsive insect cell-line are summarised. Several novel antagonists have been identified, among which the cucurbitacins are the best characterised and have been shown to compete with ecdysteroids for the ligand binding site of the insect steroid hormone receptor. Certain withanolides, limonoids and resveratrol derivatives also antagonise 20-hydroxyecdysone action. Additionally, several new phytoecdysteroids have been isolated and identified. In common with all other ecdysteriods, these possess agonistic activity in the B_{II} bioassay. Extensive SAR studies based on the potencies of a large number of purified ecdysteroids have been performed and molecular (CoMFA) modelling used to characterise ecdysteroid binding to the ligand binding site of the receptor complex.

Keywords: agonist; antagonist; bioassay; cucurbitacin; ecdysteriod; limonoid; phyto-ecdysteriod; radioimmunoassay; resveratrol; withanolide

1 INTRODUCTION

The ecdysteroid receptor complex is potentially one of the most interesting targets for the development of new classes of invertebrate pest-control agents. This derives from the essential regulatory roles of ecdysteroids at all stages of insect development and the structural dissimilarity between ecdysteroids and vertebrate steroid hormones. Ecdysteroids do not themselves have the necessary properties to be effective exogenous control agents, since they are chemically too complex, too polar and too environmentally/metabolically labile.[1] However, approximately 6% of all species of plants accumulate phyto-ecdysteroids,[2,3] analogues of the zoo-ecdysteroids, often in concentrations much higher than those found in insects or other invertebrates. To date, over 150 phyto-ecdysteroid structures have been determined.[4] Phyto-ecdysteroids are believed to contribute to the deterrence of non-adapted phytophagous invertebrates. Understanding of phyto-ecdysteroid chemistry and biochemistry can contribute to the development of new plant protection strategies by (i) enabling extensive structure–activity studies to be performed to map the ligand binding site of the receptor, followed by the design of simpler analogues and (ii) elevation of ecdysteroid levels in crop plants (most of which are ecdysteroid-negative) to provide enhanced protection. The latter strategy is facilitated by the low toxicity of ecdysteroids towards mammals.[5]

Plants, with their extensive array of secondary compounds, may be the source of other compounds which intentionally or fortuitously interact specifically with the ecdysteroid receptor complex, either as agonists or antagonists. Here, the findings of a project to determine the identities of such natural products (Fig 1) are summarised. It is hoped that these compounds will provide new probes for the investigation of the control of gene expression by ecdysteroids and that they may serve as lead compounds for the development of novel invertebrate pest-control agents.

2 METHODS

The raison d'être and overall strategy behind the project have been described recently.[3,6] Central to the success of this project has been the development of a microplate-based bioassay for ecdysteroid agonists and antagonists using the ecdysteroid-responsive

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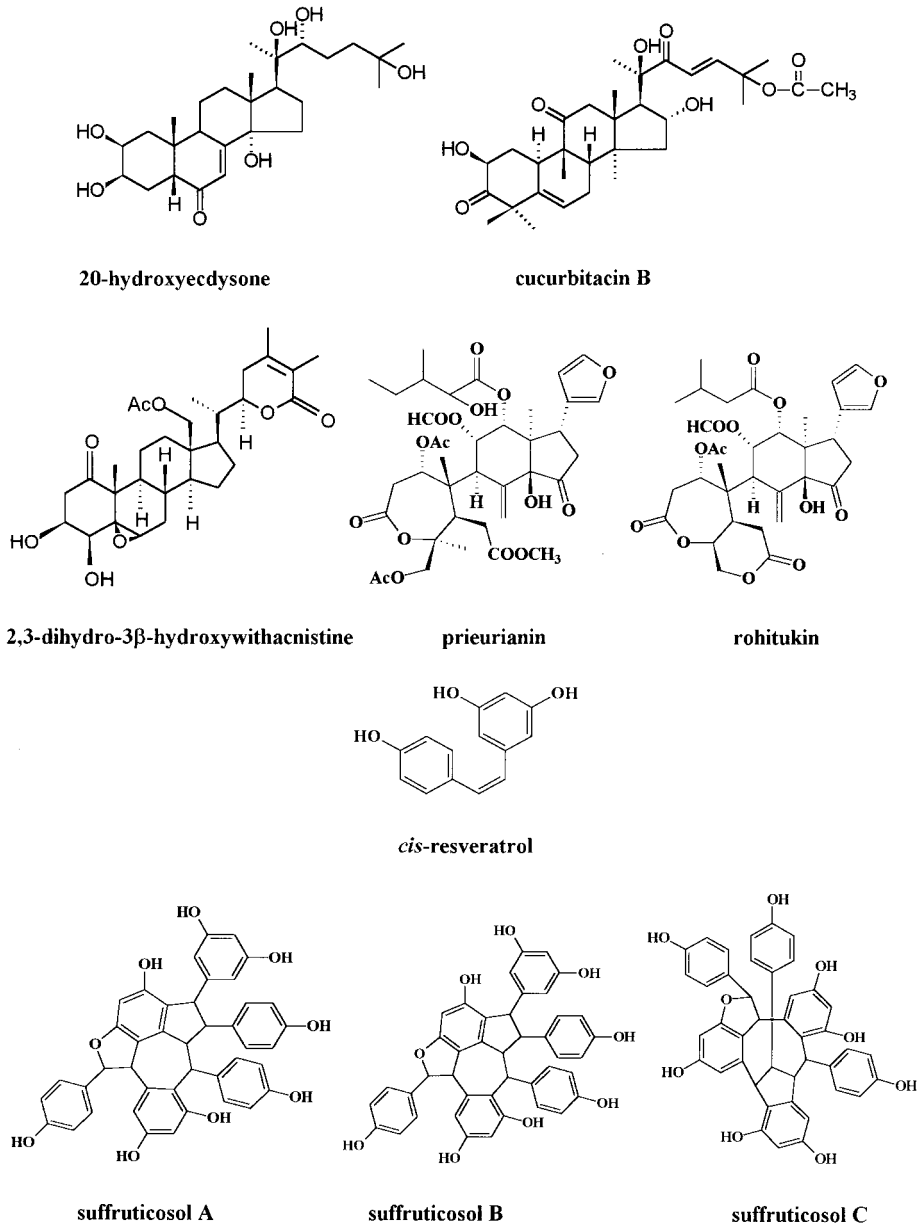

Figure 1. Structures of 20-hydroxyecdysone and selected ecdysteroid antagonists.

Drosophila melanogaster Meig B_{II} cell line.[7] Previous bioassays were too insensitive and/or cumbersome for the screening of large numbers of plant extracts. The B_{II} bioassay is simple to perform and remarkably robust, being able to cope with crude methanolic plant extracts. The bioassay also has the potential to be automated. The other important aspect to the strategy has been the miniaturisation of the sample extraction procedure, permitting the processing of many samples simultaneously. Briefly, small seed samples (*c*25 mg) are ground and then extracted with methanol (3 × 1 ml; 3 h; 55°C). The pooled extracts are mixed with water + hexane (1.3 + 2 ml). The hexane phase (containing apolar lipids and pigments) is discarded and small aliquots of the methanolic phase are assessed in the agonist and antagonist versions of the bioassay and in ecdysteroid-specific radioimmunoassays (RIAs). Most extracts (>90%) are negative and need not be considered further. RIA permits the identification of the agonist extracts which contain phyto-ecdysteroids. Portions (0.5 ml) of the interesting extracts are then separated by reversed-phase (RP)- and normal-phase (NP)-solid-phase extraction (SPE) and/or HPLC to determine the polarity and complexity of the active principles. Only if the extract is potent and appears to contain novel active components is a larger batch of seed (10 g) extracted for identification of the compounds.

3 ECDYSTEROID AGONISTS

3.1 Phytoecdysteroids

The screening of such a large number of plant species has inevitably resulted in the identification of many species which were not previously known to accumulate phytoecdysteroids. This provides basic data on the distribution of ecdysteroids in the plant world[8–19] and will, in conjunction with information

on the biology of the plant, ultimately contribute to an understanding of why a species produces particular secondary products. It is becoming clear that most (if not all) plant Families contain at least some species which accumulate ecdysteroids. Several species have been selected for further investigation and almost every one of these has yielded at least one new ecdysteroid analogue; 25-deoxy-11α,20*R*,24*S*-trihydroxyecdysone (punisterone) from *Blandfordia punicea* R Br (Blandfordiaceae),[20] 3β-xylosides of 20-hydroxyecdysone (20E) and ponasterone A from *Limnanthes douglasii* R Br (Limnanthaceae),[21] 20-hydroxyecdysone 2-β-D-glucoside from *Xerophyllum tenax* Nutt (Melanthiaceae),[22] 2-dehydro-3-*epi*-20-hydroxyecdysone from *Froelichia floridana*, (Nutt) Moq (Amaranthaceae),[23] 1α,20*R*-dihydroxyecdysone from *Axyris amaranthoides* L (Chenopodiaceae)[24] and (20*R*)22-deoxy-20,21-dihydroxyecdysone from *Rhagodia baccata* (Labill) Moq (Chenopodiaceae).[25] Although these are normally minor components of the ecdysteroid mix (and hence probably contribute little to the protection of the plant), they, together with zoo-ecdysteroids isolated from invertebrates and chemically synthesised analogues, have been particularly useful for ecdysteroid structure–activity relationship (SAR) studies in insect systems[26] and for the subsequent comparative molecular field analysis (CoMFA) modelling of ligand binding site to the *D melanogaster* ecdysteroid receptor.[27,28]

3.2 Non-steroidal agonists

Although no non-steroidal natural products with agonist activity have yet been identified with the B_{II} bioassay, others[29] have isolated 8-*O*-acetylharpagide from *Ajuga reptans* L (Labiatae) and claimed that it possesses weak agonist activity. It should, however, be mentioned that *A reptans* accumulates large amounts of ecdysteroids and a contamination of as little as 0.1% of 20E in the isolated 8-*O*-acetyl-harpagide would be sufficient to account for the observed activity.

4 ECDYSTEROID ANTAGONISTS

4.1 Cucurbitacins and cucurbitanes

Several extracts with antagonist activity have yielded cucurbitacins as the active components; thus, *Iberis umbellata* L (Cruciferae) gave cucurbitacins B and D,[30] *Cercidiphyllum japonicum* Sieb & Zucc (Cercidiphyllaceae) gave cucurbitacin D[31] and *Physocarpus opulifolius* (L) Maxim (Rosaceae) gave cucurbitacin D, together with smaller amounts of cucurbitacin F and 3-*epi*-isocucurbitacin D.[32] These compounds are all active in the micromolar range, which is well within the natural concentrations found in cucurbitacin-containing plants. Cucurbitacins B and D have been shown to displace specifically bound radiolabelled ecdysteroid from the *D. melanogaster* receptor in cell-free preparations at similar concentrations to those required to bring about antagonist response with intact cells. Preliminary SAR studies have revealed the importance of a 22-oxo-Δ^{23} functional group for antagonistic activity, since only cucurbitacins possessing this structural feature are active. Underlining this is the finding that hexanorcucurbitacin D, which lacks the C-22 to C-27 fragment of the side-chain, is actually a weak agonist.[30]

Investigation of the activities of five cucurbitanes and two cucurbitacins isolated from rhizomes of *Helmsleya carnosiflora* CY Wu & ZL Chen (Cucurbitaceae)[33] revealed that they possessed weak antagonistic activity. However, in view of the high concentrations of these compounds in the rhizomes, it is feasible that they could contribute to invertebrate deterrence.[34]

4.2 Withanolides

Fourteen withanolides which had been isolated from *Iochroma gesnerioides* (Kunth) Miers (Solanaceae)[35–37] were assessed for (ant)agonist activity. Those possessing an oxygen-containing function at C-3 (-OH or -OCH_3) and an α,β-unsaturated ketone in the side-chain ring showed antagonistic activity, with 1,2-dihydro-3β-hydroxywithacnistine being the most active (ED_{50} = 2.5×10^{-6} M versus 5×10^{-8} M 20E).[38] The importance of these two structural features is supported by studies with further purified withasteroids (Dinan L, Whiting P and Ray AB, unpublished), but more extensive SAR studies are required to assess the contributions of other structural features. An oxygen-containing function at C-3 is rare among natural withanolides and it has been suggested to be an artefact of the isolation procedure. Thus, it is not clear at present if the antagonistic activity of certain withanolides is serendipitous or whether the majority of withanolides could be activated on ingestion by insects.[38] The triterpenoid nature and structural similarity to the cucurbitacins suggest that the active withanolides also interact with the ecdysteroid binding site on the receptor.

4.3 Limonoids

Several plants of the Meliaceae have been identified as prospective sources of natural insect-control agents. Further, limonoids isolated from these species have been shown to affect insect growth and development.[39] Thus, it was not entirely surprising when extracts from several species in this Family presented antagonistic activity. The active principles in seeds of *Turraea obtusifolia* Hochst were identified as two preurianin-type limonoids (preurianin and rohitukin).[40] The potencies of the purified compounds are rather low (ED_{50} values = 10^{-5} M and 1.25×10^{-4} M, respectively) and other species in the Meliaceae are being examined for more active limonoids. In view of the considerable structural dissimilarity between the active cucurbitacins and

withanolides, on the one hand, and the active limonoids, on the other, it might be expected that they have different modes of action as antagonists.

4.4 Resveratrol and its derivatives

Paeonia suffruticosa Andrews (Paeoniaceae) is a well-known Chinese medicinal plant that was found to possess ecdysteroid antagonistic activity. Bioassay-guided HPLC purification of a methanolic extract of the seeds (4.8 g) of this species yielded *cis*-resveratrol (8.1 mg) and three novel trimers (named suffruticosol A [56 mg], suffruticosol B [74 mg] and suffruticosol C [6.2 mg]),[41] which are active as antagonists at high concentrations. However, since they are present in seeds at such high concentrations, their antagonistic activity may be biologically relevant. Detailed examination of their activity, together with that of some other related stilbenes, is in progress.

4.5 Purified natural products

The simplicity of the bioassay encouraged the assessment of the activities of a variety of purified natural products representing a range of other classes of plant secondary compounds (alkaloids, brassinosteroids, cardenolides, chromenes, glycoalkaloids, lignans, phenylpropanes, triterpenoids). Most of these were inactive (or cytotoxic at high concentrations), but several phenylpropanes and alkaloids are weak antagonists (Dinan L *et al*, unpublished). The variety of antagonistic structures is not surprising as there are many steps in the response of the B_{II} cells to 20-hydroxyecdysone where different antagonists could act; only a subset of the antagonistic compounds will interact directly with the ecdysteroid receptor complex.

5 CONCLUSIONS AND PROSPECTS

It is clear that the plant world contains several classes of natural products which can interfere specifically in the processes of ecdysteroid signal transduction in insects. Our survey of >4500 species of plants has identified many more active species than those investigated in detail so far, so further classes of active compounds can be expected. The compounds thus far identified are too chemically complex for direct use as pest-control agents, but their importance lies in their potential as lead compounds for SAR studies, as probes for the elucidation of the control gene expression by ecdysteroids and in providing new insights into insect-plant interactions.

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REFERENCES

1 Dinan L, Ecdysteroid structure and hormonal activity, in *Ecdysone*, ed by Koolman J, Georg Thieme Verlag, Stuttgart. pp 345–354 (1989).
2 Imai S, Toyosato T, Sakai M, Sato Y, Fujioka S, Murata E and Goto M, Screening results of plants for phytoecdysones. *Chem Pharm Bull* **17**:335–339 (1969).
3 Dinan L, A strategy for the identification of ecdysteroid receptor agonist and antagonists from plants. *Eur J Entomol* **92**:271–283 (1995).
4 Lafont R and Wilson ID, *The Ecdysone Handbook*, 2nd edn, The Chromatographic Society, Nottingham (1996).
5 Sláma K and Lafont R, Insect hormones–ecdysteroids: their presence and actions in vertebrates. *Eur J Entomol* **92**:355–377 (1995).
6 Dinan L, A strategy towards the elucidation of the contribution made by phytoecdysteroids to the deterrence of invertebrate predators on plants. *Russian J Plant Physiol* **45**:296–305 (1998).
7 Clément CY, Bradbrook DA, Lafont R and Dinan L, Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. *Insect Biochem Mol Biol* **23**:187–193 (1993).
8 Sarker SD, Girault J-P, Lafont R and Dinan LN, Ecdysteroids from *Gomphrena haageana* (Amaranthaceae). *Biochem System Ecol* **24**:177–178 (1996).
9 Sarker SD, Savchenko T, Whiting P, Lafont R and Dinan LN, The genus *Ourisia* (Scrophulariaceae): a potential source of phytoecdysteroids. *Biochem System Ecol*. **24**:803–804 (1996).
10 Sarker SD, Savchenko T, Whiting P, Šik V, Lafont and Dinan L, Occurrence of ecdysteroids in the genus *Centaurea* (Compositae): 20-hydroxyecdysone from *Centaurea moschata*. *Biochem System Ecol*. **25**:367–368 (1997).
11 Sarker SD, Savchenko T, Whiting P, Šik V, Underwood E and Dinan L, Analysis of species of the Ranunculaceae for ecdysteroid agonists and antagonists, I: ecdysteroids from *Pulsatilla alpina*. *Biochem System Ecol* **25**:473–474 (1997).
12 Sarker SD, Savchenko T, Šik V, Rees HH and Dinan L, 20-Hydroxyecdysone and its glucosides from *Trisetum flavescens*. *Biochem System Ecol* **26**:135–137 (1998).
13 Sarker SD, Savchenko T, Whiting P, Šik V and Dinan L, Ecdysteroids from the seeds of *Trianthema turgidifolia* and *T. pilosa* (Aizoaceae). *Biochem System Ecol* **26**:691–693 (1998).
14 Savchenko T, Whiting P, Sarker SD and Dinan L, Phytoecdysteroids in the genus *Agapanthus* (Alliaceae). *Biochem System Ecol* **25**:623–629 (1997).
15 Savchenko T, Whiting P, Sarker SD and Dinan L, Analysis of species of the Ranunculaceae for ecdysteroid agonists and antagonists II: ecdysteroids in the genus *Anemone*. *Biochem System Ecol* **26**:131–134 (1998).
16 Savchenko T, Whiting P, Sarker SD and Dinan L, Distribution and identity of phytoecdysteroids in *Gomphrena* spp. (Amaranthaceae). *Biochem System Ecol* **26**:337–346 (1998).
17 Savchenko T, Whiting P, Šik V, Underwood E, Sarker SD and Dinan L, Distribution and identities of phytoecdysteroids in the genus *Briza* (Gramineae). *Biochem System Ecol* (1998) (in press).
18 Whiting P, Savchenko T, Sarker SD, Rees HH and Dinan L, Phytoecdysteroids in the genus Limonium (Plumbaginaceae). *Biochem System Ecol* **26**:695–698 (1998).
19 Dinan L, Whiting P and Scott A, Taxonomic distribution of phytoecdysteroids in seeds of members of the Chenopodiaceae. *Biochem System Ecol* **26**:553–576 (1998).

20 Sarker SD, Dinan LN, Girault J-P, Lafont R and Waterman PG, Punisterone [(20*R*,24*S*)-25-deoxy-11α,20,24-trihydroxyecdysone): a new phytoecdysteroid from *Blandfordia punicea*. *J Nat Products* **59**:789–793 (1996).
21 Sarker SD, Girault J-P, Lafont R and Dinan LN, Ecdysteroid xylosides from *Limnanthes douglasii*. *Phytochem* **44**:513–521 (1997).
22 Alison B, Whiting P, Sarker SD, Dinan L, Underwood E, Sik V and Rees HH, 20-hydroxyedysone 2-β-D-glucopyranoside from the seeds of *Xerophyllum tenax*. *Biochem System. Ecol* **25**:255–261 (1977).
23 Sarker SD, Sik V, Rees HH and Dinan L, 2-Dehydro-3-*epi*-20-hydroxyecdysone from *Froelichia floridana* (Amaranthaceae). *Phytochem* **49**:2311–2314 (1998).
24 Sarker SD, Sik V, Rees HH and Dinan L, 1α,20*R*-Dihydroxyecdysone from *Axyris amaranthoides* (Chenopodiaceae). *Phytochem* **49**:2305–2310 (1998).
25 Dinan L, Sarker SD, Bourne P, Whiting P, Sik V and Rees HH, Phytoecdysteroids in seeds and plants of *Rhagodia baccata* (Chenopodiaceae). *Archives Insect Biochem Physiol* (1998) (in press).
26 Harmatha J and Dinan L, The biological activity of natural and synthetic ecdysteroids in the B_{II} bioassay. *Arch Insect Biochem Physiol* **35**:219–225 (1997).
27 Dinan L, Hormann RE and Fujimoto T, An extensive ecdysteroid CoMFA. *Abstracts of Papers of the American Chemical Society*, **215**:20 (1998).
28 Dinan L, Hormann RE and Fujimoto T, An extensive ecdysteroid CoMFA. *J Computer-Aided Molec Design* (1998) (in press).
29 Elbrecht A, Chen Y, Jurgens T, Hensens OD, Zink DL, Beck HT, Balick MJ and Borris R, 8-*O*-Acetylharpagide in a non-steroidal ecdysteroid agonist. *Insect Biochem Mol Biol*, **26**:519–523 (1996).
30 Dinan L, Whiting P, Girault J-P, Lafont R, Dhadialla TS, Cress DE, Mugat B, Antoniewski C and Lepesant J-A, Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. *Biochem J* **327**:643–650 (1997).
31 Sarker SD, Whiting P, Lafont R, Girault J-P and Dinan L, Cucurbitacin D from *Cercidiphyllum japonicum*. *Biochem System Ecol* **25**:79–80 (1997).
32 Sarker SD, Whiting P, Sik V, Rees HH and Dinan L, Ecdysteroid antagonists from *Physocarpus opulifolius*. *Phytochem* (1998) (in press).
33 Kasai R, Matsumoto K, Nie R-L, Morita T, Awazu A, Zhou J and Tanaka O, Sweet and bitter cucurbitane glycosides from *Helmsleya carnosiflora*. *Phytochem* **26**:1371–1376 (1987).
34 Dinan L, Whiting P, Sarker SD, Kasai R and Yamasaki K, Cucurbitane-type compounds from *Hemsleya carnosiflora* antagonise ecdysteroid action in the *Drosophila melanogaster* B_{II} cell line. *Cell Molec Life Sci* **53**:271–274 (1997).
35 Alfonso D, Kapetanidis I and Bernardinelli G, Iochromolide: a new acetylated withanolide from *Iochroma coccinenum*. *J Nat Prods* **54**:1576–1582 (1991).
36 Alfonso D, Bernardinelli G and Kapetanidis I, Withanolides from *Iochroma coccineum*. *Phytochem* **34**:517–521 (1993).
37 Alfonso D and Kapetanidis I, Withanolides from *Iochroma gesnerioides Phytochem* **36**:179–183 (1994).
38 Dinan L, Whiting P, Alfonso D and Kapetanidis I, Certain withanolides from *Iochroma gesnerioides* (Kunth) Miers (Solanaceae) antagonize ecdysteroid action in a *Drosophila melanogaster* cell line. *Entomol Exp Appl* **80**:415–420 (1996).
39 Champagne DE, Isman MB and Towers GNH, Insecticidal activity of phytochemicals and extracts of the *Meliaceae*, in *Insecticides of Plant Origin*, *ACS Symposium Series 387*, ed by Arnason. JT, Philogène BJR and Morand P. pp 96–109 (1989).
40 Sarker SD, Savchenko T, Whiting P, Sik V and Dinan LN, Two limonoids from *Turraea obtusifolia* (Meliaceae), prieurianin and rohitukin, antagonise 20-hydroxyecdysone action in a *Drosophila* cell line. *Arch Insect Biochem Physiol* **35**:211–217 (1997).
41 Sarker SD, Whiting P, Dinan L, Sik V and Rees HH, Identification and ecdysteroid antagonistic activity of three resveratrol trimers (suffruticosols A, B and C) from *Paeonia suffruticosa Tetrahedron* (1998) (in press).